Fragments of Proinsulin C-Peptide

The present invention relates to fragments of the proinsulin C-peptide, particularly N-terminal fragments, and their use in the treatment of diabetes and diabetic complications.

Insulin-dependent diabetes mellitus (IDDM), generally synonymous with type 1 diabetes, is the classical, life-threatening form of diabetes, the treatment of which was revolutionized by the discovery of insulin in 1922. The prevalence of IDDM is unfortunately widespread throughout much of the world and hence IDDM represents a serious condition with a significant drain on health resources.

The etiology of IDDM is multifactorial and not yet entirely clear. However it is characterised by a partial or complete destruction of the pancreatic beta cells. In the acute phase of IDDM insulin deficiency is thus the dominating pathophysiological feature.

After starting insulin treatment many patients enjoy good blood glucose control with only small doses of insulin. There is an early phase, the "honeymoon period", which may last a few months to a year and which probably reflects a partial recovery of beta cell function. This is, however, a temporary stage and ultimately, the progressive destruction of the beta cells leads to complete cessation of insulin secretion and increasing requirements for exogenous insulin.

While the short term effects of hypoinsulinemia in the acute phase of IDDM can be well controlled by insulin administration, the long term natural history of IDDM is darkened by the appearance in many patients of potentially serious complications known as late, or late onset complications. These include the specifically diabetic problems of nephropathy, retinopathy and neuropathy. These conditions are often referred to as

microvascular complications even though microvascular
alterations are not the only cause. Atherosclerotic
disease of the large arteries, particularly the coronary
arteries and the arteries of the lower extremities, may
also occur.

Nephropathy develops in approximately 35% of IDDM
patients, particularly in male patients and in those
with onset of the disease before the age of 15 years.
Diabetic nephropathy is characterized by persistent
albuminuria secondary to glomerular capillary damage, a
progressive reduction of the glomerular filtration rate
and eventually, end stage renal failure.

The prevalence of diabetic retinopathy is highest
among young-onset IDDM patients and it increases with
the duration of the disease. Proliferative retinopathy
is generally present in about 25% of the patients after
15 years duration and in over 50% after 20 years. The
earliest lesion of diabetic retinopathy is a thickening
of the capillary basement membrane, followed by
capillary dilatation and leakage and formation of
microaneurysms. Subsequently, occlusion of retinal
vessels occurs resulting in hypoperfusion of parts of
the retina, oedema, bleeding and formation of new
vessels as well as progressive loss of vision.

Diabetic neuropathy includes a wide variety of
disturbances of somatic and autonomic nervous function.
Sensory neuropathy may cause progressive loss of
sensation or, alternatively, result in unpleasant
sensations, often pain, in the legs or feet. Motor
neuropathy is usually accompanied by muscle wasting and
weakness. Nerve biopsies generally show axonal
degeneration, demyelination and abnormalities of the
vasa nervorum. Neurophysiological studies indicate
reduced motor and sensory nerve conduction velocities.
Autonomic neuropathy afflicts some 40% of the patients
with IDDM of more than 15 years duration. It may evolve
through defects in thermoregulation, impotence and

bladder dysfunction followed by cardiovascular reflex
abnormalities. Late manifestations may include
generalized sweating disorders, postural hypotension,
gastrointestinal problems and reduced awareness of
hypoglycemia. The latter symptom has grave clinical
implications.

A number of theories have been advanced with regard
to possible mechanism(s) involved in the pathogenesis of
the different diabetic complications but this has not
yet been fully elucidated. Metabolic factors may be of
importance and it has been shown that good metabolic
control is accompanied by significantly reduced
incidence of complications of all types. Nevertheless,
after 7-10 years of good metabolic control, as many as
15-25% of the patients show signs of beginning
nephropathy, 10-25% have symptoms of retinopathy and 15-
20% show delayed nerve conduction velocity indicating
neuropathy. With longer duration of the disease the
incidence of complications increases further. There is
thus a significant clinical need for the control and
management of these diabetic complications.

Proinsulin C-peptide is a part of the proinsulin
molecule which, in turn, is a precursor to insulin
formed in the beta cells of the pancreas. For a long
time it was believed that C-peptide (known variously as
C-peptide or proinsulin C-peptide) had no role other
than as a structural component of proinsulin,
facilitating correct folding of the insulin part.
However, it has in more recent years been recognised
that C-peptide has a physiological role as a hormone in
its own right (Wahren et al., (2000), Am. J. Physiol.
Endocrinol. Metab, 278, E759-E768). In diabetic
patients, it alleviates renal dysfunction, improves
blood flow in several tissues, ameliorates nerve
functional impairments and is believed to delay or
prevent the onset of late complications (Wahren et al.,
(2000) supra; Wahren and Johansson (1998), Horm. Metab.

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Res. 30, A2-A5). Indeed, C-peptide has been proposed
for use in the treatment of diabetes in EP 132769 and in
SE460334 for use in combination with insulin in the
treatment of diabetes and prevention of diabetic
complications.

A receptor for C-peptide has not yet been defined
but molecular studies using fluorescence correlation
spectroscopy show specific binding of human C-peptide to
cell membranes from a number of tissues (Rigler et al.,
(1999) PNAS USA 96, 13318-13323; Pramanik et al., (2001)
BBRC 284, 94-98) and intracellular calcium measurements
show that C-peptide increases the intracellular level of
calcium (Ohtomo et al., (1996) Diabetologia 39, 199-205;
Kunt et al., (1998) Diabetes 47, A30; Shafqat et al.,
(2002) Cell Mol. Life Sci. 59, 1185-1189), thus
supporting a hormone function for C-peptide.

Further work has shown that the C-terminal
pentapeptide fragment of C-peptide has similar
physiological and molecular effects to C-peptide itself,
suggesting that this segment is an essential part of C-
peptide (Wahren et al., 2000, supra; Rigler et al.,
1999, supra; Ohtomo et al., 1998, Diabetologia 41, 287-
291; Pramanik et al., 2001, supra; Shafqat et al, 2002,
supra). WO 98/13384 proposes the use of this C-terminal
pentapeptide, and other C-terminally located peptide
fragments of C-peptide in the treatment of diabetes and
diabetic complications.

However, the mechanism of action of C-peptide and
the identity of its important active sites is not
entirely clear cut. Thus, various studies suggest that
more than one signalling pathway, and perhaps even more
than one receptor, may be involved.

A number of actions of C-peptide appear to be
mediated via G-protein-coupled pathways, as indicated by
pertussis toxin inhibition of those actions. Thus,
pertussis toxin inhibits C-peptide stimulation of
$Na^+K^+ATPase$ activity, calcium influx and activation of

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MAP kinases.   Interestingly, it also interferes with C-peptide binding to cell membranes (Rigler et al., supra). Moreover, activation of protein kinase C and phosphoinositide 3-kinase P13-K) seems to be involved in C-peptide induced phosphorylation of MAP kinases (Kitamura et al., (2001), Biochem. J. 355, 123-129).

Interactions between C-peptide and receptors with catalytic activity are indicated by results showing that C-peptide attenuates protein tyrosine phosphatase activity (Li et al., (2001), B.B.R.C. 280, 615-619). Protein tyrosine phosphatases inactivate the insulin signalling pathway by dephosphorylation of the insulin receptor, insulin receptor substrates and MAP kinases. Hence, C-peptide and insulin might have a synergistic effect on the insulin signalling pathway at the level of the insulin receptor. This is further corroborated by the recent finding that C-peptide at physiological concentrations mimics insulin effects in myoblasts; it activates insulin receptor tyrosine kinase, insulin receptor substrate-1 tyrosine phosphorylation, PI3-K activity, and MAP kinase phosphorylation (Grunberger et al., (2001), Diabetologia, 44, 1247-1257). If C-peptide is added in the presence of high insulin concentrations, no further effects are observed, indicating that C-peptide and insulin may use the same signalling pathway. These authors suggested that low C-peptide levels enhance insulin effects, while at supra-physiological concentrations C-peptide blunts insulin effects. However, C-peptide, unlike insulin, does not activate Akt (protein kinase B), suggesting that C-peptide also works via mechanisms distinct from those of insulin. C-peptide-induced stimulation of glycogen synthesis in the myoblasts was blocked by Wortmannin, an inhibitor of PI3-K activity, but not by pertussis toxin (Grunberger, (2001), supra). In contrast to these findings, Zierath et al., 1996, Diabetologia 39, 421-432, found that C-peptide stimulates glucose transport in human muscle

strips, and that these effects were not mediated via the insulin receptor or tyrosine kinase activation.

It has also been speculated that C-peptide may interact with ligand-gated ion channels coupled to glutamate receptors, based, for example, on the observation that C-peptide, in common with other Glu-terminated peptides, may antagonise the N-Methyl-D-Aspartate (NDMA) receptor (Bourguignon et al., (1994), Endocrinology 134, 1589-1592). Further, free Glu has been shown to have some C-peptide activity in several assays (Johannsson et al., (2002) Biochem. Biophy. Res. Commun 295, 1035-1040). However, this has yet to be confirmed.

It has also been suggested that the effects of C-peptide may be mediated by direct membranotropic mechanisms instead of by classical receptor-ligand interactions (Ido et al., (1997), Science 277, 563-566). However, such observations are not entirely consistent with other studies showing the absence of the hallmarks of traditional pore-forming peptides in C-peptide, (Steiner et al., (1997), Science 277, 531-532; Henriksson et al., (2000), Cell Mol. Life Sci. 57, 337-342) and again this is yet to be fully resolved.

Thus, the above studies indicate that there may be a diversity of C-peptide action, with perhaps different effects being mediated in different ways. Thus, for example, receptor-mediated physiological effects may take place at or below the physiological C-peptide concentration (0.5-1.5 x 10^{-9}M), whereas supra-physiological concentrations might induce non-specific effects. Where receptor action is concerned, as mentioned above, the experimental data tends to suggest that other receptors and/or signalling pathways may be involved in addition to a G-protein coupled receptor(s). We further suggest that such diverse actions may be mediated in different ways by different portions, or segments, of the C-peptide molecule.

Thus, although clinical utility has been

demonstrated for C-peptide and its C-terminal fragment,
the above studies open up the possibility that, through
further understanding of how C-peptide works,
improvements may be achieved in C-peptide-based therapy
of diabetes and its complications, and the present
invention is directed to this aim.

Proinsulin, or large parts of it, are known in 37
different variants, representing 33 different species,
ranging from Atlantic hagfish, *Myxine glutinova*, to
human. Whilst the insulin segments (i.e. the A and B
chains of proinsulin) are well conserved between
species, C-peptide is much more highly variable, showing
not only sequence variation, but also several internal
deletions, making the length of C-peptide variable (see
Figure 1).

Human C-peptide is a 31 amino acid peptide having
the following sequence: EAEDLQVGQVELGGGPGAGSLQPLALEGSLQ
(SEQ ID. NO. 1).

C-peptide has thus hitherto been regarded as a
poorly conserved peptide. It will be seen, however,
that when different groups of C-peptide are compared,
mammalian C-peptides for example, higher levels of
conservation can be seen and conserved residues can be
identified. It will further be seen that C-peptide can
be ascribed a tripartite overall structure, with more
conserved N- and C-terminal segments and a more variable
mid-sequence, or internal, portion. Thus, in the case
of human C-peptide the N-terminal segment can be
regarded as residues 1-12, the mid-portion as residues
13-26, and the C-terminal segment as residues 27-31.

In water, C-peptide is devoid of detectable stable
secondary structure, and thus appears to be unordered.
However, under artificial conditions in trifluoroethanol
(TFE) the N-terminal 11 residues can be induced to form
an α-helical structure (Henriksson *et al.*, (2000), Cell.
Mol. Life Sci. 57, 337-342). However, no importance,
functional or otherwise, has been ascribed to this.

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As mentioned above, the C-terminal segment has been
recognised in the art to have both binding and
physiological activity, and clinical utility (see e.g.
WO 98/13384 and Ohtomo *et al*., (1998), supra). The mid-
portion also has been shown to have molecular and
physiological effects (see e.g. Ido *et al*. and Ohtomo et
al., (1998), supra) and has also been proposed in WO
98/13384 to have clinical utility. Ido *et al*., have
speculated that the mid-portion may mediate its effects
through membrane interactions, as mentioned above,
although this is still to be confirmed and rather
contradicted by other studies (Henriksson *et al*, supra).
In any event, the available data, for example showing
that the mid-portion peptide fragment comprising amino
acid residues 11-19 of human C-peptide does not displace
cell membrane-bound human C-peptide (Pramanik *et al*.,
(2001), supra), suggests that the mechanisms of the mid-
portion segment are different from those of the C-
terminal segment. Up till now, however, the N-terminal
segment (or portion) of C-peptide has not been thought
to be important for activity and indeed N-terminal
fragments or portions containing the N-terminal segment
have not been found to show any reproducible activity
(see e.g. Ohtomo *et al*., (1998), supra).

We have now surprisingly found, however, that
although not active on its own, the N-terminal segment
is functionally important, and makes an important
contribution to the activity of C-peptide. This has
been elucidated through a detailed study of the
structural basis for C-peptide activity, and has been
confirmed by experimental data showing the importance of
certain residues in the N-terminal segment for C-peptide
activity. It is now proposed, therefore, that the N-
terminal fragment has a utility in C-peptide-based
therapies, for example either in conjunction with an
active C-terminal fragment, or as part of a modified C-
peptide molecule.

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Furthermore, it has also been shown that substitution of certain amino acid residues in the N-terminal region may increase or decrease C-peptide activity. This, thus, opens up the possibility of modifying the N-terminal sequence of C-peptide in order to modulate its activity, namely the construction of peptide derivatives of the N-terminal region of C-peptide, or peptide derivatives of C-peptide (e.g. comprising modifications in the N-terminal region) which may have e.g. altered C-peptide activity or which may otherwise be useful in C-peptide based therapies.

In particular, we have now found that Glu residues 3 and 11 of human C-peptide located in the N-terminal region are particularly important for activity, in conjunction with Glu 27 of the C-terminal part, which has previously been reported to be important for activity of the C-terminal fragment and C-peptide as a whole (Pramanik et al., (2001), supra).

Whilst not wishing to be bound by theory, it is believed that these three acidic amino acid residues are important for receptor binding, and/or for C-peptide binding activities and that the reason why the C-terminal fragment is active on its own is that it can adopt a structure similar to that of the N-terminal fragment at one or both of the conserved N-terminal region Glu residues (Glu3 and/or Glu11), and thus may bind to the receptor/binding proteins not only at its own site for the C-terminal segment, but also at a site for the N-terminal segment. In this way, the C-terminal fragment can mimic the receptor interactions of the intact C-peptide.

We further find evidence that on interacting with its receptor, the N-terminal segment is induced to form an α-helical structure, and hence we now propose for the first time that such a structure is functionally important. We have found in this regard that the critical Glu residues Glu3 and Glu11, together with

other conserved amino acids in the N-terminal segment
(e.g. Gln 6 and Val 7) are located on one side of an α-
helix. We thus propose a model for receptor or protein
interaction, wherein the N-terminal segment of C-peptide
is induced into an α-helical conformation, having a
conserved surface encompassing one side of the helix and
presenting two acidic (Glu) residues for receptor or
protein binding.

In one aspect, the present invention thus provides
a peptide, being the N-terminal fragment of human
proinsulin C-peptide, and having the sequence

E   A   E   D   L   Q   V   G   Q   V   E   L   (SEQ ID. NO. 2)
1   2   3   4   5   6   7   8   9   10 11 12

or a fragment or peptide derivative thereof retaining
the functional ability of said N-terminal fragment to
contribute to C-peptide activity, wherein said fragment
or peptide derivative comprises two acidic amino acid
residues and is capable of adopting a conformation where
said two acidic amino acid residues are spatially
separated from one another by a distance of 9-14 Å
between the α-carbons thereof, preferably 10-13 Å (e.g.
10-12.6 Å); and wherein said peptide derivative is no
more than 14 amino acids in length or wherein said
derivative does not include native C-peptide of any
species (as shown in Figure 1) nor human C-peptide 1-15,
1-24 or 1-26 or rat C-peptide 1-26.

Alternatively, the spatial separation between the
two acidic residues can be considered in terms of the
number of intervening amino acids in the primary
sequence or structure. Thus, the spacing between the
two acidic amino acid residues can be 5-9 amino acid
residues, preferably 5-8 or 6-8 amino acid residues and
most preferably 7 amino acids as in SEQ. ID. NO. 2.

More particularly, the peptide fragment or
derivative is capable of adopting an α-helical

conformation, and even more particularly the two said
acidic amino acid residues are located on one side of
said α-helix. This requirement for capability of
forming an α-helix means that the peptide fragment or
derivative may form an α-helix under appropriate
conditions, for example in a suitable (e.g. structure-
promoting) solvent such as TFE (see Henriksson et al.,
supra), or when interacting with a receptor or binding
protein. This requirement may thus readily be tested
for under appropriate test conditions using standard
analytical techniques, for example NMR and circular
dichroism in TFE as described by Henriksson et al.

In a more particular embodiment, the peptide
derivative comprises also further amino acid residues of
the N-terminal fragment or segment of human C-peptide,
which are located on said one side of the said α-helix
such that said helix presents a "conserved surface"
encompassing one side of the helix. In particular, said
"conserved surface" comprises residues, in addition to
the two acidic amino acid residues (corresponding to Glu
3 and Glu 11 of human C-peptide), Gln 6 and/or Val 7 of
human C-peptide or equivalent or corresponding residues.

Preferably, the minimum length of the peptide of
the invention is 7 amino acids and the peptide is
capable of forming an α-helix. More preferably, the
minimum length is 8, 9, 10, 11 or 12 amino acids.

By "equivalent" or "corresponding" residues is
meant the residues which occur in positions equivalent
or corresponding to the stated positions, e.g. as
identified above and below, in the human C-peptide, i.e.
the equivalent or corresponding residues in other native
forms of C-peptide e.g. in other species (see e.g. Fig.
1).

Even more particularly, the two said acidic amino
acid residues of said peptide derivative are capable of
interacting with a third acidic residue (namely Glu 27)
when supplied as an additional peptide (e.g. the C-

terminal pentapeptide of human C-peptide), or indeed in
the context of a modified peptide comprising said
peptide derivative sequence and said additional peptide
sequence, in stimulating C-peptide activity. Thus, in
the context of such a modified peptide, or when said
peptide derivative is used in conjunction with said
additional peptide, the spatial separation between said
two acidic residues and said third acidic residue (e.g.
Glu 27) is in the ranges specified above, i.e. the
spatial separation is preferably equidistant between the
three acidic residues (distance between each of the
three acidic residues one with the others is the same).

The term "peptide derivative" as used herein means
that the derivative in question has a peptide structure.
In other words the derivative is a peptide derived from
or corresponding to or a variant of the N-terminal
fragment of human C-peptide having sequence
modifications as compared to the native human N-terminal
C-peptide fragment sequence. Such modifications may be
one or more amino acid deletions, additions, insertions
and/or substitutions. Chemical modification of the
peptide structure is not precluded e.g. by glycosylation
as long as the structure of the derivative remains
essentially peptide in nature. As mentioned above,
modification of an amino acid sequence may be by amino
acid substitution, for example an amino acid may be
replaced by another which preserves the physicochemical
character of the peptide (e.g. A may be replaced by G or
vice versa, V by A, L or G; E by D or vice versa; and Q
by N). Generally, the substituting amino acid has
similar properties e.g. hydrophobicity, hydrophilicity,
electronegativity, bulky side chains etc. to the amino
acid being replaced.

Additional variants may include amino and/or
carboxyl terminal fusions as well as intrasequence
insertions of single or multiple amino acids. Longer
peptides may comprise multiple copies of one or more of

the peptide sequences.

Insertional amino acid sequence variants are those
in which one or more amino acid residues are introduced
at a site in the protein. Deletional variants are
characterised by the removal of one or more amino acids
from the sequence.

The term "functional ability" as used herein means
that the fragment or derivative is capable of
functioning as the N-terminal fragment of human
proinsulin C-peptide, i.e. can substitute for the N-
terminal fragment itself, for example in the context of
the intact human C-peptide itself, or when used in
combination with the active C-terminal pentapeptide
fragment EGSLQ (SEQ ID NO. 3). Thus the fragment or
derivative retains the functional characteristics of the
N-terminal fragment, and particularly the functional
property of the N-terminal fragment in contributing to
C-peptide activity.

By "contributing" is meant that in the context of
an active C-peptide molecule (e.g. intact human C-
peptide or an active sequence-modified derivative
thereof), the substitution of the fragment/derivative of
the invention for the native N-terminal segment does not
abrogate (or substantially abrogate) C-peptide activity.
Thus, detectable, and preferably clinically or
physiologically significant levels of C-peptide activity
are retained. In other words activity is retained at
levels similar or close to levels of native C-peptide,
or any reduction in activity is such as for activity
still to be detectable, or useful, e.g. the peptide
retains at least 25% (and more preferably at least 30,
40, 50, 60, 70, 75, 80 or 90% of the activity of the
native, (e.g. "parent") molecule i.e. prior to the
substitution. Similarly, and analogously, such
contribution might be assessed in the context of
determining the activity of the N-terminal fragment or
derivative admixed with an "active" C-peptide fragment

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e.g. an active C-terminal fragment e.g. a C-terminal
pentapeptide such as the human C-terminal pentapeptide.

The term "C-peptide activity" as used herein means
any activity, exhibited by a native C-peptide, whether a
physiological response exhibited in an *in vivo* or *in
vitro* test system, or any biological activity or
reaction mediated by a native C-peptide, for example in
an enzyme assay or in binding to test tissues or
membranes.

Thus, it is known that C-peptide increases the
intracellular concentration of calcium. An assay for C-
peptide activity can thus be by assaying for changes in
intracellular calcium concentrations upon addition or
administration of the peptide (e.g. fragment or
derivative) in question. Such an assay is described in
for example in Ohtomo *et al.*, (1996), supra, Kunt *et
al.*, supra; Shafqat *et al.*, supra and in Example 1
below.

Further, C-peptide has been found to induce
phosphorylation of the MAP-kinases ERK 1 and 2 of a
mouse embryonic fibroblast cell line (Swiss 3T3), and
measurement of such phosphorylation and MAPK activation
may be used to assess, or assay for C-peptide activity,
as described for example by Kitamura *et al.*, supra and
in Example 2.

C-peptide also has a well known effect in
stimulating Na⁺K⁺ATPase activity and this also may form
the basis of an assay for C-peptide activity, for
example as described in WO 98/13384 or in Ohtomo *et al.*,
(1996), supra or Ohtomo *et al.*, (1998), supra.

An assay for C-peptide activity based on
endothelial nitric oxide synthase (eNOS) activity is
also described in Kunt *et al.*, supra, using bovine
aortic cells and a reporter cell assay.

Binding to particular cells may also be used to
assess or assay for C-peptide activity, for example to
cell membranes from human renal tubular cells, skin

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fibroblasts and saphenous vein endothelial cells using
fluorescence correlation spectroscopy, as described for
example in Rigler et al., supra, Henriksson et al.,
supra and Pramanik et al., supra. Finally, affinity
tests based on measurements of protein binding may be
used as activity tests of C-peptide.

The term "spatially separated" means the relative
3-D positions of the residues in question, i.e. the
separation, or relative positions of the residues in a
3-D structure or conformation.

It will be clear from the above that the
"fragments" of the N-terminal C-peptide fragment,
according to this aspect of the present invention, as
defined above, must comprise at least Glu 3 and Glu 11.
Such "fragments" thus include

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A  E  D  L  Q  V  G  Q  V  E  L   (SEQ ID NO. 4)
   E  D  L  Q  V  G  Q  V  E  L   (SEQ ID NO. 5)
      E  D  L  Q  V  G  Q  V  E   (SEQ ID NO. 6)
A  E  D  L  Q  V  G  Q  V  E      (SEQ ID NO. 7)
E  A  E  D  L  Q  V  G  Q  V  E   (SEQ ID NO. 8).
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However, by analogy with the active C-terminal
fragments discussed above, it is also believed that
short fragments of the N-terminal C-peptide fragments,
which contain only 1 Glu residue may be active in their
own right i.e. may exhibit C-peptide activity (i.e.
similarly to that shown for the C-terminal pentapeptide
of C-peptide). Thus, such short Glu-containing peptide
fragments may be able to bind to the receptor(s) at the
sites for both the N-terminal and C-terminal segments
and thus mimic the receptor interactions of the intact
C-peptide, as proposed for the C-terminal pentapeptide
and other C-terminal fragments above.

A further separate, but related, aspect of the
invention thus provides a peptide, being a fragment of
the N-terminal segment of a proinsulin C-peptide (namely

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the N-terminal 1-12 amino acid residues), said fragment
having no more than 6 amino acids and having C-peptide
activity. This aspect also provides such peptides for
use in therapy.

The proinsulin C-peptide may be any of the C-
peptides shown in Figure 1, but preferably will be a
mammalian C-peptide. Accordingly, such short active
peptides according to this aspect of the invention
preferably include one of the Glu residues (i.e. one of
Glu 3 and Glu 11) or corresponding or equivalent residue
in other species (preferably an acidic residue e.g.
Asp).

Representative short active fragments of this
aspect of the invention thus include fragments (i.e. C-
peptide residues) 1-6, 1-5, 1-4, 1-3, 2-7, 2-6, 2-5, 2-
4, 4-7, 3-8, 3-7, 3-6, 3-5, 6-11, 7-12, 7-11, 8-12, 8-
11, 9-12, 9-11, 10-12, particularly such fragments of
human C-peptide. Preferred such short active fragments
thus include, *inter alia*, human 3-7, E D L Q V (SEQ ID
NO. 9), human 1-5, E A E D L (SEQ ID NO. 10), human
7-11, V G Q V E (SEQ ID NO. 11).

As mentioned above, it is considered that such
short fragments have activity by virtue of including an
acidic residue important for receptor interaction and
being short enough to fit into the receptor binding
pockets for both the N- and C-terminal segments of C-
peptide. Thus, this then leads to a related, but
broader aspect of the present invention, in which it is
proposed that any short peptide (i.e. up to 6 residues
long) containing at least one acidic residue may be able
to mimic C-peptide activity, or more particularly to
mimic C-peptide receptor interactions, and thus exhibit
C-peptide activity.

In this aspect the present invention thus provides
a peptide for use in therapy, said peptide being no more
than 6 amino acid residues in length and comprising at
least one acidic amino acid residue; with the proviso

that said peptide is not ESGLQ (SEQ ID NO. 3), ELGGGP
(SEQ ID NO. 12), ELGG (SEQ ID NO. 13), ELGGG (SEQ ID NO.
14) or EVARQ (SEQ ID NO. 15).

The therapy is preferably C-peptide therapy, as
discussed further below. The peptide may be 2, 3, 4, 5
or 6 residues in length, preferably up to 5 residues
(e.g. 2 to 5 or 3 to 5). The acidic residue may be any
acidic residue as discussed further below, but
preferably will be Glu or a derivative thereof, as
discussed further below. Representative peptides of
this aspect of the invention thus include e.g.

    EEEEE (SEQ ID NO. 16);

    EEEEE   (SEQ ID NO. 17);

    EEEE   (SEQ ID NO. 18);

    EEE   (SEQ ID NO. 19) or

    EE   (SEQ ID NO. 20).

Other representative peptides may include any of
the peptides of SEQ ID NO. 16 to 20 above wherein one or
more of the E residues are replaced by Asp (D) or a Glu
derivative, or by any amino acid residue, as long as at
least one Glu (E) remains (or other acidic amino acid
residue), preferably by an amino acid residue such as
Ala (A), Leu (L), Ile (I), Ser (S), Val (V), Gln (Q),
Asn (N). Other amino acids which may be substituted
include also Gly (G).

Taken together, these various aspects of the
invention pertaining to various "active" peptides (i.e.
peptides having C-peptide activity) or peptides
contributing to C-peptide activity, can be seen to
provide a peptide for use in therapy, said peptide being
the N-terminal fragment of human proinsulin C-peptide,
and having the sequence

    E A E D L Q V G Q V E L   (SEQ ID. NO. 2)
    1 2 3 4 5 6 7 8 9 10 11 12

or a fragment or peptide derivative thereof having C-

peptide activity or retaining the functional ability of
said N-terminal fragment to contribute to C-peptide
activity, wherein said fragment or peptide derivative
comprises at least one acidic amino acid residue and
wherein said peptide derivative does not include native
C-peptide of any species (as shown in Figure 1) nor
human C-peptide 1-15, 1-24, 4-24, 7-24, 11-24, 4-31, 8-
31, 12-31 or des 13-17 (C-peptide 1-31 with residues at
positions 13-17 missing/deleted). Preferably the
peptide is capable of forming an α helix.

    This aspect also provides the use of such a peptide
in preparing a medicament for use in C-peptide based
therapy, pharmaceutical compositions containing such a
peptide and methods of treatment (i.e. C-peptide based
therapy) wherein such peptides are administered.

    The acidic amino acid residues may be the residue
of any acidic amino acid, whether naturally occurring or
synthetic. Thus, such an amino acid may be any amino
acid having a free carboxyl group (or carboxylate etc.).
This may be, for example, one of the "conventional"
acidic amino acids Glu or Asp or a non-conventional
acidic amino acid, such as are well known in the art and
widely described in the literature. Such non-
conventional acidic amino acids may include
modifications or derivatives of Asp or Glu. Acidic D-
amino acids may also be used. Representative acidic
amino acids are included in the listing of non-
conventional amino acids shown in Table 1 below.

TABLE 1

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α-aminobutyric acid	Abu	L-N-methylalanine	Nmala
α-amino-α-methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropanecarboxylate	Cpro	L-N-methylasparagine	Nmasn
aminoisobutyric acid	Aib	L-N-methylaspartic acid	Nmasp
aminonorbornylcarboxylate	Norb	L-N-methylcysteine	Nmcys
cyclohexylalanine		L-N-methylglutamine	Nmgln
cyclopentylalanine	Cpen	L-N-methylglutamic acid	Nmglu
D-alanine	Dal	Chexa L-N-methylhistidine	Nmhis
D-arginine	Darg	L-N-methylisolleucine	Nmile
D-aspartic acid	Dasp	L-N-methylleucine	Nmleu
D-cysteine	Dcys	L-N-methyllysine	Nmlys
D-glutamine	Dgln	L-N-methylmethionine	Nmmet
D-glutamic acid	Dglu	L-N-methylnorleucine	Nmnle
D-histidine	Dhis	L-N-methylnorvaline	Nmnva
D-isoleucine	Dile	L-N-methylornithine	Nmorn
D-leucine	Dleu	L-N-methylphenylalanine	Nmphe
D-lysine	Dlys	L-N-methylproline	Nmpro
D-methionine	Dmet	L-N-methylserine	Nmser
D-ornithine	Dorn	L-N-methylthreonine	Nmthr
D-phenylalanine	Dphe	L-N-methyltryptophan	Nmtrp
D-proline	Dpro	L-N-methyltyrosine	Nmtyr
D-serine	Dser	L-N-methylvaline	Nmval
D-threonine	Dthr	L-N-methylethylglycine	Nmetg
D-tryptophan	Dtrp	L-N-methyl-t-butylglycine	Nmtbug
D-tyrosine	Dtyr	L-norleucine	Nle
D-valine	Dval	L-norvaline	Nva
D-α-methylalanine	Dmala	α-methyl-aminoisobutyrate	Maib
D-α-methylarginine	Dmarg	α-methyl-y-aminobutyrate	Mgabu
D-α-methylasparagine	Dmasn	α-methylcyclohexylalanine	Mchexa
D-α-methylaspartate	Dmasp	α-methylcylcopentylalanine	Mcpen
D-α-methylcysteine	Dmcys	α-methyl-α-napthylalanine	Manap
D-α-methylglutamine	Dmgln	α-methylpenicillamine	Mpen

D-α-methylhistidine	Dmhis	N-(4-aminobutyl)glycine	Nglu
D-α-methylisoleucine	Dmile	N-(2-aminoethyl)glycine	Naeg
D-α-methylleucine	Dmleu	N-(3-aminopropyl)glycine	Norn
D-α-methyllysine	Dmlys	N-amino-α-methylbutyrate	Nmaabu
D-α-methylmethionine	Dmmet	α-napthylalanine	Anap
D-α-methylornithine	Dmorn	N-benzylglycine	Nphe
D-α-methylphenylalanine	Dmphe	N-(2-carbamylethyl)glycine	Ngln
D-α-methylproline	Dmpro	N-(carbamylmethyl)glycine	Nasn
D-α-methylserine	Dmser	N-(2-carboxyethyl)glycine	Nglu
D-α-methylthreonine	Dmthr	N-(carboxymethyl)glycine	Nasp
D-α-methyltryptophan	Dmtrp	N-cyclobutylglycine	Ncbut
D-α-methyltyrosine	Dmty	N-cycloheptylglycine	Nchep
D-α-methylvaline	Dmval	N-cyclohexylglycine	Nchex
D-N-methylalanine	Dnmala	N-cyclodecylglycine	Ncdec
D-N-methylarginine	Dnmarg	N-cylcododecylglycine	Ncdod
D-N-methylasparagine	Dnmasn	N-cyclooctylglycine	Ncoct
D-N-methylaspartate	Dnmasp	N-cyclopropylglycine	Ncpro
D-N-methylcysteine	Dnmcys	N-cycloundecylglycine	Ncund
D-N-methylglutamine	Dnmgln	N-(2,2-diphenylethyl)glycine	Nbhm
D-N-methylglutamate	Dnmglu	N-(3,3-diphenylpropyl)glycine	Nbhe
D-N-methylhistidine	Dnmhis	N-(3-guanidinopropyl)glycine	Narg
D-N-methylisoleucine	Dnmile	N-(1-hydroxyethyl)glycine	Nthr
D-N-methylleucine	Dnmleu	N-(hydroxyethyl))glycine	Nser
D-N-methyllysine	Dnmlys	N-(imidazolylethyl))glycine	Nhis
N-methylcyclohexylalanine	Nmchexa	N-(3-indolylyethyl)glycine	Nhtrp
D-N-methylornithine	Dnmorn	N-methyl-γ-aminobutyrate	Nmgabu
N-methylglycine	Nala	D-N-methylmethionine	Dnmmet
N-methylaminoisobutyrate	Nmaib	N-methylcyclopentylalanine	Nmcpen
N-(1-methylpropyl)glycine	Nile	D-N-methylphenylalanine	Dnmphe
N-(2-methylpropyl)glycine	Nleu	D-N-methylproline	Dnmpro
D-N-methyltryptophan	Dnmtrp	D-N-methylserine	Dnmser
D-N-methyltyrosine	Dnmtyr	D-N-methylthreonine	Dnmthr
D-N-methylvaline	Dnmval	N-(1-methylethyl)glycine	NvalNma
γ-aminobutyric acid	Gabu	N-methyla-napthylalanine	nap
L-t-butylglycine	Tbug	N-methylpenicillamine	Nmpen
L-ethylglycine	Etg	N-(p-hydroxyphenyl)glycine	Nhtyr
L-homophenylalanine	Hphe	N-(thiomethyl)glycine	Ncys

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L-α-methylarginine	Marg	penicillamine	Pen
L-α-methylaspartate	Masp	L-α-methylalanine	Mala
L-α-methylcysteine	Mcys	L-α-methylasparagine	Masn
L-α-methylglutamine	Mgln	L-α-methyl-t-butylglycine	Mtbug
L-α-methylhistidine	Mhis	L-methylethylglycine	Metg
L-α-methylisoleucine	Mile	L-α-methylglutamate	Mglu
L-α-methylleucine	Mleu	L-α-methylhomophenylalanine	Mhphe
L-α-methylmethionine	Mmet	N-(2-methylthioethyl)glycine	Nmet
L-α-methylnorvaline	Mnva	L-α-methyllysine	Mlys
L-α-methylphenylalanine	Mphe	L-α-methylnorleucine	Mnle
L-α-methylserine	Mser	L-α-methylornithine	Morn
L-α-methyltryptophan	Mtrp	L-α-methylproline	Mpro
L-α-methylvaline	Mval	L-α-methylthreonine	Mthr
N-(N-(2,2-diphenylethyl) carbamylmethyl)glycine	Nnbhm	L-α-methyltyrosine	Mtyr
		L-N-methylhomophenylalanine	Nmhphe
1-carboxy-1(2,2-diphenyl-ethylamino)cyclopropane	Nmbc	N-(N-(3,3-diphenylpropyl) carbamylmethyl)glycine	Nnbhe

Preferably the acidic amino acid is Glu or a derivative thereof, e.g. γ-carboxy Glu (Gla), L-N-methyl Glu (Nmglu), L-α-methyl Glu (MGlu).

In a preferred embodiment, the peptide derivatives of the present invention have the formula (I):

$$X_n - Y - X_m - Y - X_p \qquad (I)$$

wherein

   X is any amino acid;

   Y is an acidic amino acid;

   n = 0-6;

   m = 1-12; and

   p = 0-6.

Preferably, the peptide of the invention will have the formula (I), wherein m = 5-8 or 5-9.

X may be any amino acid, whether natural or synthetic, conventional or non-conventional. In addition to the well known 20 conventional amino acids (Ala(A); Cys(C); Asp(D); Glu(E); Phe(F); Gly(G); His(H);

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Ile(I); Lys(K); Leu(L); Met(M); Asn(N); Pro(P); Gln(Q);
Arg(R); Ser(S); Thr(T); Val(V); Trp(W); and Tyr(Y)), a
number of non-conventional amino acids are shown in
Table 1 above.

In particular X may be Ala, Leu or Glu, or any
other helix promoting amino acid, or any derivative
thereof, e.g. norleucine (Nle).

Further, assuming that the two Y residues in
Formula I above correspond to Glu3 and Glu11 of human C-
peptide, X may also be any of the amino acid residues
which occur in the corresponding positions of human or
other (preferably mammalian) C-peptide.

More particularly, in X_n, X may be Glu(E), Gln(Q),
Asp(D), Asn(N), Ala(A), Val(V), Leu(L), Ser(S), Ile(I)
or Gly(G) or a derivative thereof, although preferably
at least one X will be a helix promoting amino acid,
preferably Ala(A), Leu(L), Nle or Glu(E).

In X_m, X may likewise be selected from Glu(E),
Gln(Q), Asp(D), Asn(N), Ala(A), Leu(L), Ile(I), Ser(S),
Val(V) or Gly(G), but preferably at least one, and
preferably at least two, three or four X will be a helix
promoting amino acid, preferably Ala(A), Leu(L), Nle or
Glu(E).

In X_p, X may similarly be selected from Glu(E),
Gln(Q), Asp(D), Asn(N), Ala(A), Leu(L), Ile(I), Ser(S),
Val(V) or Gly(G), and preferably at least one X will be
a helix-promoting amino acid, preferably Ala(A), Leu(L),
Nle or Glu(E).

Y may be any acidic amino acid, as defined both
generally, and more particularly, above. As mentioned
above in preferred embodiments Y is preferably Glu(E) or
a derivative thereof.

n is preferably 0-5, 1-5, 1-4, 1-3 or 1-2; e.g. 0,
1, 2, 3, 4, 5 or 6;

m is preferably 1-10, 1-8, 2-8, 3-8, 4-8, 5-8, 5-9
or 6-8; for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or
12;

p is preferably 0-5, 0-4, 0-3, 0-2 or 1-5, 1-4, 1-3 or 1-2, e.g. 0, 1, 2 or 3, 4, 5 or 6.

In more particular embodiments group (X_n) may be $Y(X_n)$ where Y is any acidic amino acid as defined above, preferably Glu(E), X is as defined above (both particularly and generally) and n is 0-6, preferably 1-3, most preferably 1.

Group (X_n) may thus preferably be E-A, E-V-, E-L or E-E, or E-Nle-.

Group (X_m) may be

$$X_{(1-4)}-Q-V-X_{(1-4)}, \quad \text{e.g.} \quad X_{(2)}-Q-V-X_{(3)};$$
$$X_{(1-4)}-Q-X_{(1-5)}, \quad \text{e.g.} \quad X_{(2)}-Q-X_{(4)};$$
$$X_{(1-4)}-Q-A-X_{(1-4)}, \quad \text{e.g.} \quad X_{(2)}-Q-A-X_{(3)};$$
$$X_{(1-4)}-Q-L-X_{(1-4)}, \quad \text{e.g.} \quad X_{(2)}-Q-L-X_{(3)};$$
$$X_{(1-4)}-Q-E-X_{(1-4)}, \quad \text{e.g.} \quad X_{(2)}-Q-E-X_{(3)};$$
$$X_{(1-3)}-L-Q-X_{(1-5)}, \quad \text{e.g.} \quad X-L-Q-X_{(4)};$$

wherein X is as defined above, both particularly and generally.

Group (X_p) may preferably be L, I, V, A, S, E, Q , D, N or G or Nle, preferably A, E, L or Nle, and especially preferably L or Nle.

A representative peptide derivative of the invention may thus be of Formula (II), (III), (IV) or (V):

$$\text{E A (Y) (X) L Q (X)(X) Q (X) (Y) L} \quad \text{(II)}$$
$$\text{E A (Y) (X) L Q (V)(X) Q (X) (Y) L} \quad \text{(III)}$$
$$\text{(X) (X) (Y) (X) L Q (X) (X) (X) (X) (Y) L} \quad \text{(III)}$$
$$\text{E (X) (Y) (X) L Q (X) (X) (X) (X) (Y) L} \quad \text{(IV)}$$

where (X) is any amino acid as defined above (both generally and particularly) and Y is any acidic amino acid, preferably Glu(E) or a derivative thereof, especially Glu(E).

In Formula II, III, IV or V, X is preferably Ala

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(A), Leu(L), Glu(E) or Nle, or any derivative thereof or other helix promoting amino acid, especially preferably Ala(A), Leu(L), Glu(E) or Nle.

Representative peptides derivatives of the invention thus include:

```
E A E A L Q A A Q A E L                    (SEQ ID NO. 21)
E A E E L Q E E Q E E L                    (SEQ ID NO. 22)
E A E L L Q L L Q L E L                    (SEQ ID NO. 23)
E A E (Nle) L Q (Nle) (Nle) Q (Nle) E L    (SEQ ID NO. 24)
  A E A L Q A A Q A E L                    (SEQ ID NO. 25)
    E A L Q A A Q A E L                    (SEQ ID NO. 26)
  A E E L Q E E Q E E L                    (SEQ ID NO. 27)
    E E L Q E E Q E E L                    (SEQ ID NO. 28)
  A E L L Q L L Q L E L                    (SEQ ID NO. 29)
    E L L Q L L Q L E L                    (SEQ ID NO. 30)
  A E(Nle) L Q(Nle) (Nle) Q(Nle) E L       (SEQ ID NO. 31)
    E(Nle) L Q(Nle) (Nle) Q(Nle) E L       (SEQ ID NO. 32)
E A E A L Q A A Q A E A                    (SEQ ID NO. 33)
E A E A L Q A A Q A E                      (SEQ ID NO. 34)
  A E A L Q A A Q A E                      (SEQ ID NO. 35)
    E A L Q A A Q A E                      (SEQ ID NO. 36)
E A E A L Q A A Q A E E                    (SEQ ID NO. 37)
E A E A L Q A A Q A E (Nle)                (SEQ ID NO. 38)
  A E A L Q A A Q A E (Nle)                (SEQ ID NO. 39)
    E A L Q A A Q A E (Nle)                (SEQ ID NO. 40)
```

Representative derivatives may also include the non-human counterparts of the C-peptide N-terminal segment in other species as shown in Figure 1, particularly other mammalian species.

The length of the peptide derivatives of the invention is not critical and may, for example, lie in the region of up to 30 residues e.g. up to 26, 24, 20, 16 or 12 residues. Such peptide derivatives may be from e.g. 5, 6, 7 or 8 amino acid residues in length.

Representative peptide lengths thus include e.g. 6-26,
6-20, 6-12, 8-12 etc.

Amino acid replacement studies have demonstrated
that residues in the N-terminal fragment of C-peptide
may be important for activity and that activity may be
modulated (e.g. decreased or increased) by substituting
certain residues. Thus, activity of human C-peptide may
be increased or remain by substituting any of Asp 4, Val
7, Gly 8 and Val 10 with Ala. Preferred peptide
derivatives of the invention thus include peptides of
SEQ ID NO. 2, wherein one or more of D4, V7, G8 and V10,
particularly D4, G8 and V10, are replaced by A.

Further studies have shown that substituting the
same residues with other amino acids, in particular
helix non-promoters may decrease activity. Thus
activity of human C-peptide may be decreased by
substituting any of the above residues with Pro.
Further preferred peptide derivatives thus include
peptides of SEQ ID NO. 2 wherein one or more of D4, V7,
G8 and V10 (particularly D4, G8 and V10) are replaced by
P or other amino acid residues which do not promote
secondary structure e.g. G. Peptides having decreased
C-peptide activity may be of value in certain
situations. This, thus, opens up the possibility of
modulating C-peptide activity by designing peptide
derivatives with appropriate substitutions.

As mentioned above, aside from the short active
family of peptides and fragments discussed above, the N-
terminal fragment and peptide derivatives of the
invention as defined above are not active on their own,
but contribute to C-peptide activity, e.g. are active in
the context of a modified peptide comprising a C-peptide
C-terminal portion as required for activity. In other
words the N-terminal fragment and peptide derivative
sequences may "substitute for" the corresponding native
N-terminal segment (sequence) in a native C-peptide
molecule. Thus, modified "C-peptides" (or C-peptide

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sequences or polypeptides) may be constructed which
comprise the N-terminal fragment or peptide derivatives
of the invention, along with other portions or segments
of a C-peptide molecule (e.g. the mid-portion and/or C-
terminal segment) and which exhibit C-peptide activity.

Thus, a further aspect of the invention provides a
peptide comprising the N-terminal fragment of human
proinsulin C-peptide, or a fragment or peptide
derivative thereof as defined above, and having C-
peptide activity, but not including native C-peptide of
any species (as shown in Figure 1) nor human C-peptide
1-15, 1-24 or des 13-17.

More particularly, this aspect of the invention
provides a peptide having an amino acid sequence
comprising (i) the N-terminal fragment of human insulin
C-peptide having the sequence

    E A E D L Q V G Q V E L   (SEQ ID NO. 2)

or (ii) a fragment or peptide derivative of amino
acid sequence SEQ ID NO. 2 retaining the functional
ability of said N-terminal fragment to contribute to C-
peptide activity, wherein said fragment or peptide
derivative comprises two acidic amino acid residues and
is capable of adopting a conformation wherein said two
acidic amino acid residues are spatially separated from
one another by a distance of 9-14 Å between the α-
carbons thereof, preferably 10-13 Å (e.g. 10-12.6 Å);

said peptide having C-peptide activity, but not
including native C-peptide of any species (as shown in
Figure 1) nor human C-peptide 1-15, 1-24 or des 13-17
(C-peptide 1-31 but with residues at positions 13-17
missing).

The spacing between the two acidic amino acids can
be 5-9 amino acid residues, preferably 5-8 or 6-8 amino
acid residues and most preferably 7 amino acid residues.
As indicated above, the peptide is preferably capable of

forming an α helix.

C-peptide activity is as defined above.

It will be seen, therefore, that this aspect of the
invention provides modified "C-peptides" which have C-
peptide activity.

Thus, in this aspect of the invention, an N-
terminal C-peptide fragment or derivative may be
combined with e.g. the C-terminal pentapeptide of human
C-peptide (or its counterpart from other species) to
provide a modified C-peptide having C-peptide activity.

Thus, in one such embodiment the N-terminal
fragment or derivative of the invention as defined
above, may be linked to a C-peptide C-terminal fragment
or sequence by a spacer sequence. Such a spacer
sequence may comprise all or a portion of a C-peptide
mid-portion sequence (the mid-portion being residues 13
to 26 of human C-peptide, or the corresponding segment
of another native C-peptide molecule; see Figure 1) e.g.
a truncated mid-portion sequence, a modification or
derivative of the mid-portion sequence, e.g. having
amino acid substitutions, or a synthetic sequence. Such
a synthetic sequence will preferably be composed of
amino acids which do not promote secondary structure,
e.g. Gly, Pro, Ser, Ile, Val, Asp, Asn and Gln. Gly is
preferred. However any amino acid may be used including
e.g. Ala or Leu which are helix-promoting. Such a
synthetic sequence may be a homopolymeric sequence, e.g.
poly Gly. The spacer may also comprise a portion of
another segment of the C-peptide molecule. The length
of the spacer sequence is not critical (as indeed is its
presence).

Accordingly, this may be e.g. up to 15 residues
long, preferably 0 to 12, 0 to 10, 0 to 8, 0 to 6, 0 to
5, 0 to 4 or 0 to 3 residues (e.g. 1 to 10, 1 to 18, 1
to 6, 1 to 4, or 1 to 3; or 2 to 8, 2 to 6 or 2 to 4).

Thus, a representative spacer sequence may include
various truncations and "deletions" of the human C-

peptide mid-portion segment, e.g. human C-peptide 13-17
and 23-26; human C-peptide 13-15 and 23-26; human C-
peptide 23-26; human C peptide 23-26 wherein residues 23
and 24 are substituted by Ala. Also included are
homopolymers of e.g. Gly having from 1 to 15, e.g. 1 to
12, 1 to 8, 1 to 6 or 1 to 3 residues, or spacers
comprising homopolymeric sequences (e.g. poly Gly)
coupled to other sequences or residues, e.g. Gly_{12}-AL
etc.

The C-terminal segment or sequence may be any C-
peptide C-terminal segment or sequence having C-peptide
activity e.g. human 27-31 (or its counterpart in other
species - see Fig. 1) or a fragment or derivative
thereof (see e.g. WO 98/13384).

In another particular embodiment of this aspect,
the invention provides a modified mammalian C-peptide
which comprises one or more sequence modifications in
the N-terminal segment thereof, but wherein amino acid
residues Glu3 and Glu11 (or residues corresponding
thereto) are conserved.

In such an embodiment, the N-terminal segment may
be regarded as residues 1 to 12 of a native C-peptide
sequence. In particular, Gln 6 (or a corresponding
residue) is also conserved. Leu 5 (or a corresponding
residue) may also be conserved. Furthermore, Glu1
and/or Leu 12 (or corresponding residues) may also be
conserved.

In particular Asp 4, Val 7, Gly 8 and/or Val 10, or
positions corresponding thereto, particularly Asp 4, Gly
8 and/or Val 10 may be modified, for example by
replacement with Ala (and/or other amino acids as
discussed above e.g. Glu, Leu and Nle). Other residues
which may be modified include Glu 1, Ala 2 and/or Gln 9
or their counterparts. Such a modified C-peptide will
have C-peptide activity, as defined and discussed above.

Representative examples of such peptides according
to these various aspects of the invention thus include:

EAEDLQVGQVELGGGGGGGGGGGGGGEGSLQ (human 1-12-$(G)_{14}$-human 27-31) (SEQ ID NO. 41)

EAEDLQVGQVELGGGGGGEGSLQ (human 1-12-$(G)_6$-human 27-31) (SEQ ID NO. 42)

EAEDLQVGQVELGGGGGGGGGGGGALEGSLQ (human 1-12-$(G)_{12}$-human 25-31) (SEQ ID NO. 43)

EAEDLQVGQVELGGGPGPLALEGSLQ (human 1-12, 13-17, 23-31) (SEQ ID NO. 44)

EAEDLQVGQVELGGGPLALEGSLQ (human 1-12, 13-15, 23-31) (SEQ ID NO. 45)

EAEDLQVGQVELLEGSLQ (human 1-12, 26-31) (SEQ ID NO. 46)

EAEDLQVGQVELEGSLQ (human 1-12, 27-31) (SEQ ID NO. 47)

EAEDLQVGQVELAAALEGSLQ (human 1-12; AA, human 25-31) (SEQ ID NO. 48)

EAEDLQVGQVELGGGGGGGGGGGGGGEVARQ (human 1-12-$(G)_{14}$ Rat 27-31) (SEQ ID NO. 49).

Further representative examples include any of the N-terminal peptide derivatives of SEQ ID NO. 21 to 40 above coupled either directly or via any of the spacer sequences exemplified above (e.g. in SEQ ID NOS. 39 to 46 or 48-49) to human 27-31.

Such modified peptides may also include chimeric C-peptides comprising portions or segments of C-peptide sequence from different species e.g. human 1-12, 13-26, rat 27-31; or human 1-12, rat 13-26, rat 27-31.

Indeed, also may be included chimeric C-peptides wherein different portions or segments of the same C-peptide molecule are combined in non-native order and/or numbers, e.g. human C-peptide (27-31)-(6-12)-(27-31).

The modified peptides of this aspect of the invention preferably do not include the A and/or B chains of proinsulin.

The peptides and fragments and peptide derivatives mentioned above, all subsumed under the general heading of "peptides of the invention" may readily and conveniently be synthesised using known and standard techniques such as are widely known and well described

in the literature. Suitable methods include e.g. the
well known Merrifield solid phase synthesis method and
derivatives thereof.

Also included in the invention are the salts,
solvates and esters of the peptides, such as may be
prepared and used in accordance with standard
pharmaceutical procedures well known in the art.

Thus the peptides of the invention may be presented
as pharmaceutically or physiologically acceptable salts
e.g. acid addition salts. This may include both organic
and inorganic salts such as those prepared for example
from acids such as hydrochloric, hydrofluoric, sulfuric,
sulfonic, tartaric, fumaric, hydrobromic, glycolic,
citric, maleic, phosphoric, succinic, acetic, nitric,
benzoic, ascorbic, p-toluenesulfonic, benzene-sulfonic,
naphthalenesulfonic, propionic, and the like.
Preferably, the acid addition salts are those prepared
from hydrochloric acid, acetic acid, or succinic acid.
Such salts may be prepared by conventional methods well
known to those skilled in the art.

Alternatively the peptide may be converted into a
carboxylic acid salt, such as an ammonium or alkali
metal salt e.g. a sodium, potassium or lithium salt etc.

As mentioned above, the peptides of the invention
have a utility in C-peptide based therapies, that is in
the therapy of (i.e. in combatting) any condition which
may be alleviated or improved by, or which responds to,
C-peptide administration. "Therapy" and "combatting" in
this regard include both treatment and prophylaxis. In
particular the peptides of the invention (i.e. including
the peptide fragments, peptide derivatives and modified
peptides defined above etc.) can be used for the therapy
of (i.e. for combatting) diabetes and diabetic
complications, most notably type 1 diabetes and its
complications. As used herein the term "diabetic
complications" includes all complications which may be
associated with various forms of diabetes, in particular

retinopathy, neuropathy and nephropathy. The peptides
may thus be used in treatment of type 1 diabetes
patients with one or more of the above-mentioned
complications, or for preventing or retarding the
development of such complications. Thus, the peptides
may be used in C-peptide replacement therapy of diabetic
patients. As discussed above, the peptides of the
invention which are not in themselves active may be used
in conjunction with active peptides such as C-peptide
itself or a C-terminal fragment of a C-peptide e.g. the
C-terminal pentapeptide of human C-peptide. As
discussed above, C-peptide has diverse actions which may
be mediated through diverse mechanisms. By combining
different modified C-peptides or C-peptide fragments in
this manner, different effects may be obtained.

A further aspect of this invention thus provides a
peptide of the invention as hereinbefore defined, for
use in therapy, and in particular in C-peptide based
therapy, (e.g. C-peptide replacement therapy in
diabetes), and also the use of such a peptide in
preparing a medicament for use in C-peptide based
therapy (e.g. for combatting diabetes or diabetic
complications).

More particularly, this aspect of the invention
provides a peptide, being the N-terminal fragment of
human insulin C-peptide, and having the sequence

        E   A   E   D   L   Q   V   G   Q   V   E   L   (SEQ ID. NO. 2)
        1   2   3   4   5   6   7   8   9 10 11 12

or a fragment or peptide derivative thereof retaining
the functional ability of said N-terminal fragment to
contribute to C-peptide activity, wherein said fragment
or peptide derivative comprises two acidic amino acid
residues and is capable of adopting a conformation where
said two acidic amino acid residues are spatially
separated from one another by a distance of 9-14 Å

between the α-carbons thereof, preferably 10-13 Å (e.g. 10-12.6 Å), wherein said derivative does not include native C-peptide of any species (as shown in Figure 1) nor human C-peptide 1-15 or 1-24, for use in therapy, and the use of a such peptide in preparing a medicament for use in C-peptide based therapy. The spacing between the two acidic amino acids can be 5-9 amino acid residues, preferably 5-8 or 6-8 amino acid residues and most preferably 7 amino acid residues. Preferably the peptide is capable of forming an α helix.

This aspect of the invention further provides a peptide having an amino acid sequence comprising (i) the N-terminal fragment of human insulin C-peptide having the sequence

E A E D L Q V G Q V E L   (SEQ ID NO. 2)

or (ii) a fragment or peptide derivative of amino acid sequence SEQ ID NO. 2 retaining the functional ability of said N-terminal fragment to contribute to C-peptide activity, wherein said fragment or peptide derivative comprises two acidic amino acid residues and is capable of adopting a conformation where said two acidic amino acid residues are spatially separated from one another by a distance of 9-14 Å between the α-carbons thereof, preferably 10-13 Å (e.g. 10-12.6 Å);

said peptide having C-peptide activity, but not including native C-peptide of any species (as shown in Figure 1) nor human C-peptide 1-15, 1-24 or des 13-17 for use in therapy, and the use of such a peptide in preparing a medicament for use in C-peptide-based therapy. The spacing between the two acidic amino acids can be 5-9 amino acid residues, preferably 5-8 or 6-8 amino acid residues and most preferably 7 amino acid residues. As indicated above, the peptide is preferably capable of forming an α helix.

Alternatively viewed, this aspect of the invention

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also provides a method of combatting diabetes or
diabetic complications in a patient, said method
comprising administering to said patient a peptide of
the invention, as defined above.

A further aspect of the invention provides a
pharmaceutical composition comprising a peptide of the
invention, as defined above, together with at least one
pharmaceutically acceptable carrier or excipient.

In this aspect, the peptide of the invention may be
a peptide, being the N-terminal fragment of human
insulin C-peptide, and having the sequence

         E   A   E   D   L   Q   V   G   Q   V   E   L   (SEQ ID. NO. 2)
         1   2   3   4   5   6   7   8   9   10 11 12

or a fragment or peptide derivative thereof retaining
the functional ability of said N-terminal fragment to
contribute to C-peptide activity, wherein said fragment
or peptide derivative comprises two acidic amino acid
residues and is capable of adopting a conformation where
said two acidic amino acid residues are spatially
separated from one another by a distance of 9-14 Å
between the α-carbons thereof, preferably 10-13 Å (e.g.
10-12.6 Å) and wherein said derivative does not include
native C-peptide of any species (as shown in Figure 1)
nor human C-peptide 1-15 or 1-24; or it may be a peptide
having an amino acid sequence comprising (i) the N-
terminal fragment of human insulin C-peptide having the
sequence

         E A E D L Q V G Q V E L   (SEQ ID NO. 2)

or (ii) a fragment or peptide derivative of amino
acid sequence SEQ ID NO. 2 retaining the functional
ability of said N-terminal fragment to contribute to C-
peptide activity, wherein said fragment or peptide
derivative comprises two acidic amino acid residues and

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is capable of adopting a conformation where said two
acidic amino acid residues are spatially separated from
one another by a distance of 9-14 Å between the α-
carbons thereof, preferably 10-13 Å (e.g. 10-12.6 Å);

said peptide having C-peptide activity, but not
including native C-peptide of any species (as shown in
Figure 1) nor human C-peptide 1-15, 1-24 or des 13-17.
The spacing between the two acidic amino acids can be 5-
9 amino acid residues, preferably 5-8 or 6-8 amino acid
residues and most preferably 7 amino acid residues.
Preferably, the peptide is capable of forming an α
helix.

As mentioned above, in the case of the peptides of
the invention which are not themselves active they may
be combined with active peptides. Thus such a
pharmaceutical composition may comprise in addition a C-
peptide or C-peptide fragment having C-peptide activity,
such as C-terminal fragments of C-peptide, in particular
the human C-terminal pentapeptide. Suitable fragments
are further defined in WO98/13384. However, the
respective peptides need not both be included in the
same composition/medicament and could be administered
separately, in separate compositions/ medicaments,
simultaneously or sequentially. The two fragments may,
however, form part of a single molecule.

A further aspect of the invention thus provides a
product containing a peptide, being the N-terminal
fragment of human insulin C-peptide, and having the·
sequence

      E   A   E   D   L   Q   V   G   Q   V   E   L   (SEQ ID. NO. 2)
      1   2   3   4   5   6   7   8   9   10 11 12

or a fragment or peptide derivative thereof retaining
the functional ability of said N-terminal fragment to
contribute to C-peptide activity, wherein said fragment
or peptide derivative comprises two acidic amino acid

residues and is capable of adopting a conformation where
said two acidic amino acid residues are spatially
separated from one another by a distance of 9-14 Å
between the α-carbons thereof, preferably 10-13 Å (e.g.
10-12.6 Å) and wherein said derivative does not include
native C-peptide of any species (as shown in Figure 1)
nor human C-peptide 1-15 or 1-24, together with a
peptide having C-peptide activity (particularly a C-
terminal C-peptide fragment, e.g. the human C-peptide C-
terminal pentapeptide SEQ ID NO. 3) as a combined
preparation for simultaneous, separate or sequential use
in C-peptide based therapy (e.g. in combatting diabetes
and/or diabetic complications). The spacing between the
two acidic amino acids can be 5-9 amino acid residues,
preferably 5-8 or 6-8 amino acid residues and most
preferably 7 amino acid residues. Preferably, the
peptide is capable of forming an α helix.

The peptides may also be used in combination or
conjunction with other agents active or effective to
treat diabetes and/or its complications. Such other
active agents include for example insulin. In such
"combination" therapies the peptide(s) and second active
agent may be administered together in the same
composition or separately in separate compositions,
simultaneously or sequentially.

A further aspect of the invention thus provides a
product containing a peptide of the invention as
hereinbefore defined, together with a further active
agent effective to combat diabetes or diabetic
complications, as a combined preparation for
simultaneous, separate or sequential use in combatting
diabetes and/or diabetic complications.

Preferably such a further active agent is insulin.

In such combined therapies, where insulin is used,
it is to be understood that the term "insulin"
encompasses all forms, types and derivatives of insulin
which may be used for therapy e.g. synthetic, modified,

or truncated variants of the active human insulin
sequence.

The compositions of the invention may be
administered in any convenient way, e.g. orally or
parenterally, for example by the subcutaneous,
intramuscular or intravenous route. The compositions of
this invention may comprise active peptides of the
invention, together with a pharmaceutically acceptable
carrier therefor and optionally, other therapeutic
ingredients, for example human insulin. The total
amount of active ingredients in the composition may vary
from 99.99 to 0.01 percent of weight. The carrier must
be acceptable in the sense that it is compatible with
other components of the composition and is not
deleterious to the recipient thereof.

The compositions may be formulated according to
techniques and procedures well known in the art and
widely described in the literature, and may comprise any
of the known carriers, diluents or excipients. Thus,
for example, compositions of this invention suitable for
parenteral administration conveniently comprise sterile
aqueous solutions and/or suspensions of the
pharmaceutically active ingredients (e.g. the peptides
of the invention) preferably made isotonic with the
blood of the recipient, generally using sodium chloride,
glycerin, glucose, mannitol, sorbitol, and the like. In
addition, the compositions may contain any of a number
of adjuvants, such as buffers, preservatives, dispersing
agents, agents that promote rapid onset of action or
prolonged duration of action and the like.

Compositions of this invention suitable for oral
administration may, for example, comprise the peptides
in sterile purified stock powder form preferably covered
by an envelope or envelopes (enterocapsule) protecting
from degradation (dicarboxylation or hydrolysis) of the
peptides in the stomach and thereby enabling absorption
of these substances from the gingiva or in the small

intestine. The envelope(s) may contain any of a number
of adjuvants such as buffers, preservative agents,
agents that promote prolonged or rapid release giving an
optimal bioavailability of the compositions in this
invention, and the like.

Appropriate representative formulations (i.e.
compositions) and dosages etc. are described in WO
98/13384.

The invention will now be described in more detail
in the following non-limiting Examples, and with
reference to the drawings in which:

Figure 1 is an alignment showing all reported C-
peptide amino acid sequences.

Figure 2 shows the ability of the various C-peptide
mutants from Example 4 to phosphorylate MAPK.

Figure 3 shows the ability of C-peptide to
phosphorylate MAPK when residues 4, 7, 8 and 10 are all
altered to either Ala or Pro, where Ala may keep the
tendency of the native C-peptide tendency to induce α-
helix formation and induce α-helix formation and Pro may
prevent α-helix formation.

EXAMPLES

Example 1
Intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) Measurements as
an Assay of C-peptide Activity

Based on studies showing a C-peptide effect on the
intracellular Ca^{2+} concentration ($[Ca^{2+}]$) (Ohtomo et al.,
supra, Kunt et al., supra), an assay has been developed,
for C-peptide activity, based on measuring $[Ca^{2+}]_i$
changes in human renal tubular cells, namely a rise in
$[Ca^{2+}]_i$.

Peptides

The following peptides were used, all prepared by solid-
phase peptide synthesis and purchased from Sigma
(Genosys (Cambridge, UK): human C-peptide, scrambled
C-peptide (with the same residues, but randomly ordered
des(27-31) C-peptide and C-terminal pentapeptide
(EGSLQ).

Cell culture

Human renal tubular cells were obtained from the outer
cortex of renal tissues obtained from non-diabetic
patients undergoing elective nephrectomy for renal cell
carcinomas.   The collection of the tissue samples during
surgery was approved by the Ethics Committee of the
Karolinska Hospital.   The cells were cultured in RPMI
1640 (Life Technologies, Grand Island, N.Y.)
supplemented with 10% fetal calf serum, 2 mM L-
glutamine, 10 mM HEPES, benzylpenicillin (100 U/ml), and
streptomycin (100 U/ml).   Cells were grown to confluency
on cover slips in a six-well plate and starved in serum-
free medium for 4 hours before experiments.

Measurements of $[Ca^{2+}]_i$

Changes in $[Ca^{2+}]_i$ were measured on cells attached to
cover slips.   The cells were loaded with 2 μM of the
fluorescent Ca^{2+} indicator fura-2/AM at 37°C for 30
minutes in a buffer containing 115 mM NaCl, 24 mM $NaHCO_3$,
4.7 mM KCl, 1.26 mM $CaCl_2$ 1.2 mM KH_2PO_4, 1.2 mM $MgSO_2$ 11.1
mM glucose, 20 mM HEPES, and 5 mg/ml bovine serum
albumin, pH 7.4.   Cover slips were mounted as the bottom
of an open chamber placed on a thermostatically
controlled stage of an inverted epifluorescence
microscope (Zeiss, Axiovert 135).   The microscope was
connected to a SPEX fluorolog-2 system for dual-wave-
length excitation fluorimetry.   The emissions of the two
excitation wavelengths of 340 and 380 nm were used to
calculate the fluorescence ratio (F340/F380) reflecting

changes in $[Ca^{2+}]_i$, [17]. Treatments during recordings
were made using a perifusion system attached to the
chamber. Changes in $[Ca^{2+}]_i$ were measured using an
attached CCD camera that allowed recording of individual
cells in the cell clusters. The influence of pertussis
toxin was studied by preincubation of the cells with the
toxin at 1 μg/ml for 4 h at 37°C.

Results

Human renal tubular cells loaded with the indicator
fura-2/AM were exposed in different series of
experiments to the peptides studied. Changes in $[Ca^{2+}]_i$
were recorded. The effect of 5 nM human C-peptide may
be seen both as a F340/F380 ratio trace and as images of
the cells taken at different time points. The most
reproducible responses were obtained at 5 and 10 nM.
Hence, comparative measurements were performed at these
peptide concentrations. Results similar to those with
human C-peptide were obtained with the C-terminal
pentapeptide of the human C-peptide, while scrambled C-
peptide and des(27-31) C-peptide, lacking the C-terminal
pentapeptide part of C-peptide, gave no effect.
Successive exposures of the same cells to 5 nM of human
C-peptide, a scrambled C-peptide, and the C-terminal
pentapeptide, EGSLQ, establish the specificity of the C-
peptide effect.

Preincubation of the cells with pertussis toxin at 1
μg/ml eliminated the responses with both C-peptide and
the C-terminal pentapeptide. This confirms that the Ca^{2+}
effect is dependent on G-protein-coupled signal
transduction pathways. When cells were perfused with a
buffer without Ca^{2+}, the effects of the peptides were
abolished, indicating that extracellular Ca^{2+} is required
for the response.

Example 2

<u>Western Blot Analysis of MAPK Activation as an Assay of</u>
<u>C-peptide Activity</u>

<u>Cell culture and treatments:</u>
We have used HRTC (human renal tubular cells) isolated
in our laboratory from outer cortex of renal tissue
obtained from patients undergoing elective nephrectomy.
Cells were cultivated in RPMI medium supplemented with
10% FBS, glutamine, Hepes and penicillin streptomycin.
We have always used P3 (passage 3). After cells were
grown to confluence, they were cultured in serum-free
medium for 2-24 h to render them to be quiescent. The
cells were then treated with either C-peptide, or
controls with vehicle at 37°C. Subsequently, the cells
were washed twice with PBS and lysed in ice-cold lysis
buffer (50 mM Hepes the lysate was kept on ice foe 30 mm
and centrifugated at 12000xg for 10 mm. The supernatant
was stored at -80°C. Protein concentration were
determined by the method of Lowry using bovine serum
albumin as standard.

<u>Western Blot analysis:</u>
Was performed using 2.5-25 ug protein per lane in 10%
SDS gel depending on which protein we want to detect.

Example 3

<u>Experiments showing functional importance of Glu 3, Glu</u>
<u>11 and Glu 27 in Human C-peptide</u>

The peptides used, as shown in Table 2, below were
prepared by solid phase peptide synthesis. In these
peptides each or all of Glu 3, Glu 11 and Glu 27 of
human C-peptide were replaced by Ala. In addition, all
of Glu 3, Glu 11 and Glu 27 were replaced by Gln.

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The peptides were tested for C-peptide activity by
monitoring for rises in $[Ca^{2+}]_i$ as described in Example
1. The following results were obtained:

Table 2

Peptide	C-peptide activity in $[Ca^{2+}]_i$ assay
Glu 3 Ala	-
Glu 11 Ala	+/-
Glu 27 Ala	+/-
Glu 3, 11, 27 Ala	-
Glu 3, 11, 27 Gln	-

The results confirm the essentiality of Glu 27 as
reported in Pramanik et al., supra and establish the
essentiality of Glu 3 and Glu 11.

Example 4
Importance of glutamic acid residues within the
C-peptide primary structure for its biological activity

Swiss 3T3 cells, a rat fibroblast cell line, passage 6,
were seeded 96 h before use in three 6-well plates.
After 96 h, the cells in each well were confluent. The
cultivation medium was Dulbecco's Modified Eagle Medium
(DMEM) without sodium pyruvate, with pyridoxineHCl, 4500
mg/l glucose, 50 μg/ml gentamicin, and 10% foetal calf
serum (FCS).

The cells were washed thoroughly with 37°C starvation
medium (cultivation medium as above without addition of
FCS) and then kept in 1 ml 37°C starvation medium for
2.5 h. 15 min. prior to the experiment, the buffer was
changed to 37°C Hanks' buffer (Gibco), and then kept in
37°C.

Human C-peptide and the four mutants (i) $E_3 \rightarrow A_3$, (ii)
$E_{11} \rightarrow A_{11}$ (iii) $E_{27} \rightarrow A_{27}$ and (iv) $E_3 \rightarrow A_3$, $E_{11} \rightarrow A_{11}$, $E_{27} \rightarrow A_{27}$ were
diluted to 1 nM in Hanks' buffer.

The cells in the wells were stimulated for 3 minutes at
37°C with 1 ml each of the 1 nM peptide solutions. As a
negative control, certain wells were treated with Hanks'
buffer only. Each peptide solution and the negative
control were applied in three separate wells.

Following stimulation of the cells with the peptide
solutions, the cells were washed 3 times with ice-cold
Hanks' buffer. All buffer in the well was removed, and
the cells were lysed with 200 μl ice-cold lysis buffer
(50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM
sodium pyrophosphate, 2 mM NaVO$_3$, 1 x Complete™ protease
inhibitor, and 1% (v/v) Nonidet P40).

The cell lysates were kept in the dishes on ice for 30
min. The lysate was removed with a rubber policeman,
transferred to Eppendoff tubes, homogenised (vortex),
and kept on ice for 60 min. The tubes were vortexed and
centrifuged for 15 min at 16 000 x g in the cold room.
The supernatants were transferred into new Eppendorf
tubes and kept on ice.

Protein content was determined in the collected
supernatants using a BCA Protein Assay Reagent Kit
(Pierce), including albumin standards. The collected
samples had a protein concentration in the range of 806-
1071 μg/ml. Samples for SDS-PAGE were prepared by
standardizing the protein content to 600 μg/ml in a SDS-
PAGE sample buffer. Prepared samples (10 μl, 6 μg
protein) were subjected to SDS-PAGE in 10% Tris-glycine
gels (Novex) and transferred to 0.2 μm PVDF membranes
(Novex) using standard procedures. The membranes were
blocked with 5% fat-free milk powder in TBS/T (Tris-

buffered saline + 0.1% Tween-20) for 1 h at room
temperature.

The PVDF membranes were incubated with primary
antibodies against (I) Phosphop44/42 MAP Kinase
(Thr202/Tyr204) and (II) p44/42 MAP Kinase,
respectively. The Phospho-p44/42 MAP Kinase
(Thr202/Tyr204) antibody detects p42 and p44 MAP kinase
(Erk1 and Erk2) only when catalytically activated by
phosphorylation at Thr202 and Tyr204 of human Erk, or
Thr183 and Tyr185 of rat Erk. Both antibodies are
commercially available, polyclonal antibodies from
rabbits (Cell Signalling Technology, #9101 and #9102,
respectively). Following incubation with the primary
antibodies, diluted 1:1000, in TBS/T overnight at 4°C on
a rocking table, the PVDF membranes were rinsed and
incubated with a secondary antibody, (goat anti-rabbit
IgG (H+L) HRP (horseradish peroxidase) conjugate,
diluted 1:20000) in TBS/T for 1 h at room temperature on
a rocking table. The membranes were rinsed with TBS/T,
and HRP activity on the membranes was detected with the
SuperSignal® West Pico Chemiluminescent Substrate kit
(Pierce).

Photographic films were exposed to the membranes, and
bands of appropriate sizes on the resulting films were
quantitated by densitometry, using a GS-710 Imaging
Densitometer (Bio-Rad) and Quantity One software (Bio-
Rad). The averaged results are displayed in Fig. 2.

Notably, substitution of any one of the three glutamic
acid residues at positions 3, 11, and 27 results in a
decreased phosphorylation of p44/42 MAP kinase by the
corresponding C-peptide mutant. The decrease in
phosphorylation is indicative of a decreased biological
activity. Possibly, the glutamic acid residues at
positions 11 and 27 are more important than the glutamic

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acid residue at position 3. Consequently, the mutant
carrying three mutations ($E_3 \rightarrow A_3$, $E_{11} \rightarrow A_{11}$, $E_{27} \rightarrow A_{27}$) also
displays a loss of phosphorylating activity in this
assay.

Example 5

Importance of N-terminally located amino acid residues
for potential C-peptide secondary structure

The following peptides were analysed by circular
dichroism (CD) spectroscopy (data not shown) in 10 mM
phosphate buffer, pH 7, and in 50% (vol/vol)
trifluoroethanol in phosphate buffer, pH 7:

C-peptide	EAEDLQVGQV ELGGGPGAGS LQPLALEGSL Q
Alanine-rich C-peptide	EAEALQAAQA ELGGGPGAGS LQPLALEGSL Q
Proline-rich C-peptide	EAEPLQPPQP ELGGGPGAGS LQPLALEGSL Q

The resulting CD spectra reveal that all three peptides
lack secondary structure in phosphate buffer. In the
presence of 50% trifluoroethanol, C-peptide has a
tendency to form a partially α-helical structure. This
tendency is even more pronounced in the alanine- rich C-
peptide, while the lack of secondary structure remains
in the proline-rich C-peptide.

Trifluoroethanol disfavors intramolecular hydrogen
binding between peptide and solvent, and can be employed
to study the tendency of a peptide to adopt a stable
secondary structure, e.g. as a part of a protein. These
results also imply that the induced α-helix is formed in
the first ten amino acid residues, since proline
substitutions can prevent α-helix formation, and alanine
substitutions can further induce α-helix formation.

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Example 6

Importance of N-terminally located amino acid residues for C-peptide biological activity

Swiss 3T3 cells, passage 6, were seeded 96 h before use in three 6-well plates. After 96 h, the cells in each well were nearly confluent. The cultivation medium was Dulbecco's Modified Eagle Medium (DMEM) without sodium pyruvate, with pyridoxineHCl, 4500 mg/l glucose, 50 μg/ml gentamicin and 10% foetal calf serum (FCS).

The cells were washed thoroughly with 37°C starvation medium (cultivation medium as above without addition of FCS) and then kept in 1 ml 37°C starvation medium for 2.5 h. Prior to the experiment, the buffer was changed to 37°C Hanks' buffer (Gibco), and then kept in 37°C.

The cells in the wells were stimulated for 3 minutes at 37°C with 2 ml each of the peptides of Example 5, peptide concentration 1 nM. As a negative control, certain wells were treated with Hanks' buffer only. Each peptide solution and the negative control were applied in three separate wells.

Following stimulation of the cells with the peptide solutions, the cells were washed with ice-cold Hanks' buffer. All buffer in the well was removed, and the cells were lysed with 200 μl ice-cold lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10mM sodium pyrophosphate, 2 mM NaVO$_3$, 1 x Complete^{TM} protease inhibitor, and 1% (v/v) Nonidet P40).

The cell lysates were kept in the dishes on ice for 80 min. The lysate was removed with a rubber policeman, transferred to Eppendoff tubes, homogenised (vortex), and kept on ice for 30 min. The tubes were vortexed and centrifuged for 15 minutes at 16 000 x g the cold room.

The supernatants were transferred into new Eppendorf
tubes, kept on ice.

Protein content was determined in the collected
supernatants using a BCA Protein Assay Reagent Kit
(Pierce), including albumin standards. The collected
samples had a protein concentration in the range of 3
28-667 μg/ml. Samples for SDS-PAGE were prepared by
standardizing the protein content to 248 μg/ml in a SDS-
PAGE sample buffer. Prepared samples (approximately 5
μg protein) were subjected to SDS-PAGE, transferred to
PVDF membranes, and the membranes were subjected to
primary and secondary antibodies, as outlined in Example
1. HRP activity on the membranes was detected by
chemoluminescense, and photographic films were exposed
to the membranes. Bands of appropriate sizes on the
resulting films were quantitated by densitometry, using
a GS-710 Imaging Densitometer (BioRad) and Quantity One
software (Bio-Rad). The results are displayed in Fig 3.

In this assay, substitution of native residues for
alanine residues in positions 4, 7, 8, and 10 did not
decrease the biological activity of C-peptide, measured
as capacity of phosphorylating p44/42 MAP kinase.
However, substitution of native residues for proline
residues in positions 4, 7, 8, and 10 resulted in a
decrease in phosphorylating capacity of C-peptide.
Therefore the capacity for form an N-terminally situated
helix is important for full C-peptide activity.